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A 24-Amino-Acid Polypeptide Is Essential for the Biosynthesis of the Coenzyme Pyrrolo-Quinoline-Quinone

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At least four genes are required for the biosynthesis of the coenzyme pyrrolo-quinoline-quinone (PQQ) in *Acinetobacter calcoaceticus*. The DNA region where one of these genes was mapped codes for a polypeptide of only 24 amino acids. Here we show that indeed this small peptide is essential for PQQ synthesis. Site-directed mutagenesis shows that at least one glutamate and one tyrosine residue of the polypeptide are essential for its function.

Pyrrolo-quinoline-quinone (PQQ) is used by a variety of bacteria as a coenzyme in dehydrogenase reactions catalyzed by the so-called quinoproteins. Quinoproteins that have been described are glucose dehydrogenase (2, 4, 5), alcohol dehydrogenase (1, 12), and methanol dehydrogenase (3, 8). Some bacterial species that contain quinoproteins synthesize the coenzyme, whereas others such as *Escherichia coli* (9) and *Acinetobacter lwoffi* (16) are dependent on PQQ from an outside source. Labelling experiments revealed that two amino acids, glutamate and tyrosine, serve as building blocks for PQQ biosynthesis (10, 11, 15).

From *Acinetobacter calcoaceticus* we have cloned and sequenced a 5,000-bp DNA fragment containing four genes involved in PQQ synthesis (6, 7). Expression of these genes in *E. coli* led to the production of the coenzyme in this organism (6), indicating that the four genes contain all the necessary information for PQQ synthesis. Three of the *pqq* genes code for proteins with M_rs of 29,700, 10,800, and 43,600. In the region where the fourth *pqq* gene (gene IV) was mapped, only two possible small reading frames could be found, transcribed from opposing strands and encoding polypeptides of 24 (upper strand) and 22 (lower strand) amino acids (Fig. 1). Introduction of a Tn*5* insertion (Tn*5*-18) (Fig. 1) in the chromosome of *A. calcoaceticus* through homologous recombination completely abolished PQQ synthesis (6), showing the importance of this DNA region.

For further analysis of the gene IV region, more Tn*5* insertions were isolated by the method described previously (6). The locations of the insertions were determined by DNA sequencing (Fig. 1). Plasmids containing the insertion mutants were tested for the ability to complement for PQQ synthesis in an *A. calcoaceticus* strain that contains the Tn*5*-18 insertion in the chromosome. The activity of the PQQ-dependent enzyme glucose dehydrogenase was used as an indicator of PQQ synthesis (7). Tn*5*-27, which, like Tn*5*-18, is located within both putative reading frames (Fig. 1), also abolishes PQQ synthesis. Insertion Tn*5*-110, which is outside the reading frames but within a region containing an 11-bp perfect inverted repeat (Fig. 1), did not affect PQQ production. Apparently, this inverted repeat is not important for gene IV function. Tn*5*-118, which is also outside both putative reading frames, did abolish PQQ production. This could indicate that gene IV function is not related to one of these reading frames. Tn*5* insertions, however, can also block transcription. Therefore, if the Tn*5*-118 insertion is located between the promoter and the translation start of a reading frame, it would also prevent synthesis of the corresponding polypeptide. To test this, we deleted the internal *Hpa*I fragment of the Tn*5*-118 insertion, thereby removing most of this Tn*5* element (13). The resulting plasmid complemented again for PQQ synthesis, showing that the Tn*5*-118 insertion has an effect on sequences downstream and is therefore probably located in the leader sequence. Since the 24-amino-acid-encoding open reading frame is located downstream of Tn*5*-118, this reading frame is a very likely candidate for gene IV function.

Finally, more direct evidence that a 24-amino-acid peptide is the gene IV product came from the introduction of a point mutation in the start codon (ATG → ATA) by oligo-directed mutagenesis by the method of Kunkel (14). The other 22-amino-acid reading frame remained unaltered by this mutation (Fig. 1). Indeed, the resulting mutant could no longer complement for PQQ synthesis.

In what way could a 24-amino-acid polypeptide be involved in PQQ synthesis? Its small size makes a direct enzymatic function in the conversion of glutamate and tyrosine to PQQ unlikely. A regulatory role of the gene IV product in the expression of the other *pqq* genes is also improbable, since previous experiments already showed that gene IV is also essential for PQQ synthesis in an *E. coli* strain (6). In these experiments, the expression of the *pqq* genes from *A. calcoaceticus* was under control of the *E. coli lac* promoter, which excludes a transcriptional control of these genes by the gene IV peptide. Therefore, it is likely that the small polypeptide has a more direct role in synthesis of the coenzyme. One possibility is that either one or both precursors of PQQ (glutamate and tyrosine) are used not directly as free amino acids but as part of the gene IV polypeptide. Since indeed both amino acids are present in the polypeptide (glutamate at positions 16 and 22 and tyrosine at position 20), we tested the importance of these residues for gene IV activity. Using oligo-directed mutagenesis (14), we created three different base pair substitution mutants: the A's at positions 908, 919, and 926 were substituted for by T residues, leading to mutations of Glu-16 → Asp, Tyr-20 → Phe, and Glu-22 → Asp, respectively (Fig. 1). The Glu-22 → Asp mutation had no effect on PQQ synthesis, but the two other amino acid substitutions completely abol-

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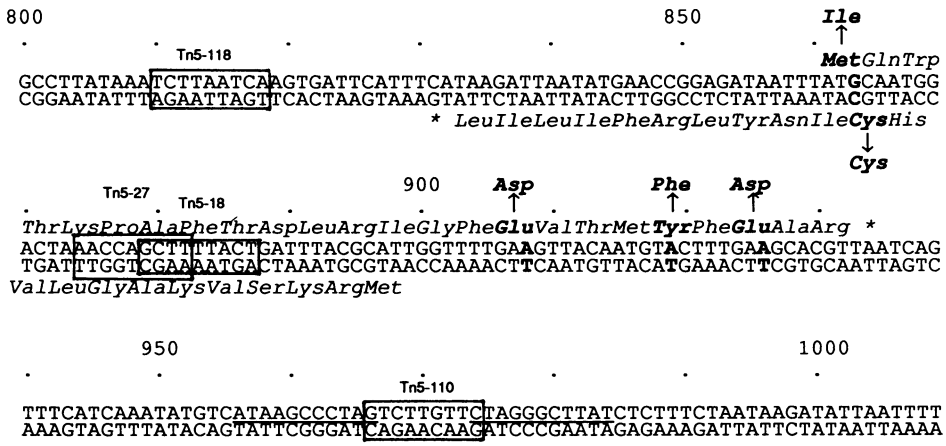

FIG. 1. Nucleotide sequence of the gene IV region. The insertion sites of the different Tn*5* insertions are indicated by boxes representing the nine base pairs that are duplicated upon Tn*5* insertion. The amino acid sequences of the two possible open reading frames are indicated with italics. The inverted repeat sequences are underlined. Base pairs that have been mutated (and the corresponding amino acid residues) are indicated in boldface.

ished production of the coenzyme, indicating the importance of the Glu-16 and Tyr-20 residues. Although it is obvious that mutation of these two residues could also affect other putative functions of the gene IV product, our results are in agreement with the possibility that one or both of these amino acids are used as precursors for PQQ. To test this hypothesis further, in vitro experiments using the purified gene IV peptide and the enzymes of the PQQ pathway have to be done.

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